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COMPARISON OF A RAPID METHOD OF COUNTING BACTERIA IN MILK WITH THE STANDARD PLATE METHOD*

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Having described a rapid method of counting bacteria in milk by means of "lilliputian" plate cultures,¹ I propose here to discuss the results obtained by this method in the examination of a series of milks during the past year. The methods employed will again be described, since they differ essentially from those previously used in milk analyses.

THE RAPID METHOD OF COUNTING BACTERIA IN MILK

In the usual, or standard, method of milk analysis, a very small fraction of a cubic centimeter of milk is plated, and incubated long enough for the bacteria to grow into colonies visible to the naked eye. In my method a comparatively low dilution of milk² is made with nutrient agar, spread over a definite area on a microscopic glass slide, and incubated only until the small colonies are visible under a compound microscope. These little colonies are then rendered prominent and easy to count by the following method of staining. The culture is dried down, fixed in the flame, treated with 10% acetic acid in alcohol to prevent the agar from firmly binding the stain, and stained with a 1:4 dilution of Loeffler's methylene blue, applied for 2 minutes. The stain is then partially washed out in alcohol or water and the preparation dried for examination. The colonies are a deep blue, while the agar background is only tinged or quite clear. (Fig. 1).

The counting is done under the low power of a compound microscope, a 2/3 or 1/2 inch (16 or 12 mm.) objective and an eyepiece of medium power.

To determine the area of the microscopic field the diameter is measured with a stage micrometer and the formula πR^2 applied. The area of the little plate is 400 sq. mm. (2 by 2 cm.), and the area of the microscopic field 2 mm. with a common combination of lenses. Thus the area of the plate becomes 200 times that of the microscopic field. The number of colonies in a field of the microscope, multiplied by 200, and again multiplied by the dilution of the milk used in making the plates, gives the number of bacteria per cubic centimeter of milk. At least 20 fields should be counted and these should be selected mechanically to avoid errors proceeding from the personal equation.

ANALYTIC RESULTS

Comparative analyses were made on 37 milks. Most of the samples came from the university creamery or the university barn. At the

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¹ Science, 1915, 42, p. 255. Jour. Am. Med. Assn., 1916, 66, p. 889.

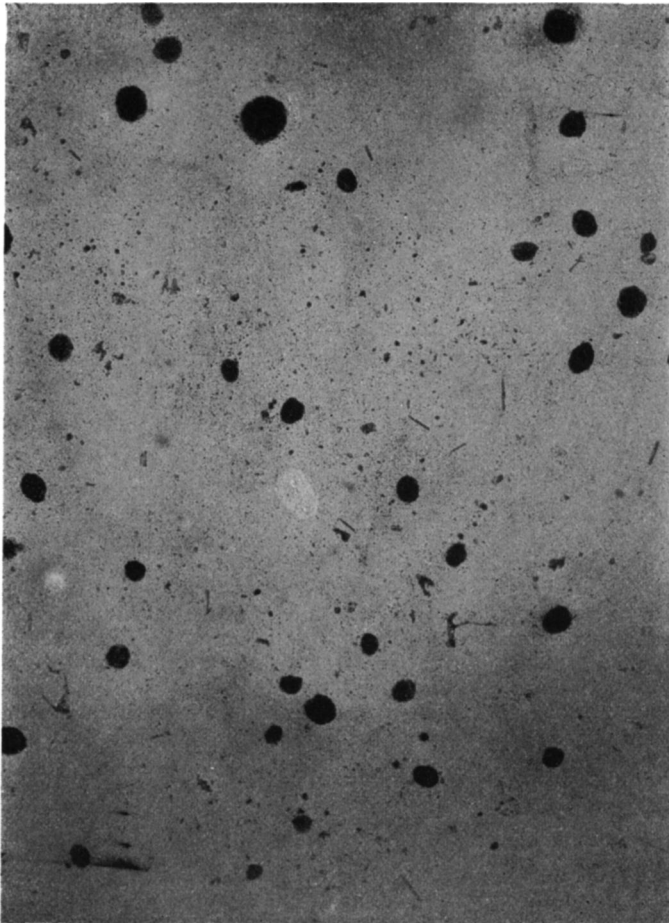


Fig. 1. A microscopic field showing colonies on the little plates after incubation period of $4\frac{1}{2}$ hours. $\times 30$.

creamery the milk was obtained directly from the farmers, some of it having been brought a considerable distance. In some cases it was examined immediately upon arrival, in which case it represented ordinary market milk, and in other cases it stood about the laboratory some hours before analysis, with the result that its bacterial count was high. The milk from the university barn, as it was produced under good conditions, usually had a low bacterial content. Single or at most a few samples of other milks were used from time to time as occasion offered.

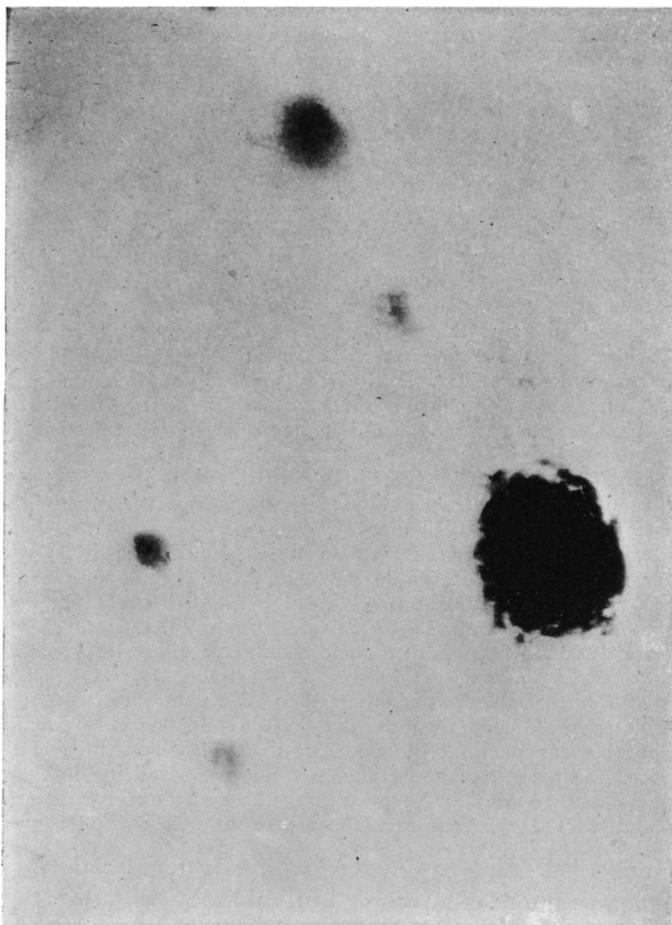


Fig. 2. Microscopic field showing a few of the same colonies seen in Fig. 1. $\times 225$.

Parallel analyses were usually made by the same person, but in some cases the standard plates were made by others and the little plates by ourselves.

Methods used.—The plate cultures were made in the usual way according to the standard methods. The milk was first thoroughly shaken, and then 1 c.c. was removed for analysis. The water blanks, made from tap water sterilized in the autoclave, contained either 9 or 99 c.c. The agar in my own work was made according to the standard methods and had a reaction of +1. The period of incubation was 48 hours at 37 C. In most of the samples the counting was done in 3 ways; first, with the naked eye, again, under a reading glass,

and finally, with the lens suggested by the committee on milk, of the American Public Health Association.² Whenever possible all the colonies on the plate were counted. Several dilutions were always made and usually all dilutions were counted, and the results averaged.

The little plates were made by putting 1 c.c. of the milk, after thorough shaking, into the agar directly, or after dilution with sterile tap water. The dilutions most frequently employed were 1:20 and 1:200. Attention is called to the fact that these small dilutions are used even with highly contaminated milks.

The counts of the little plates were made under 3 different magnifications—with a 16-mm. lens, a 6-mm. (1/4 in.) lens, and a 2-mm. lens. Several plates were usually counted for each sample.

A marked difference existed between the counts obtained under different magnifications. With the lowest power some of the minute colonies were missed, while with the higher dry power all the colonies were seen, as well as groups of dead bacteria which were not readily distinguished from colonies of growing bacteria. With the oil-immersion objective, all the groups of bacteria were counted, these counts naturally being higher than those secured by either of the other methods.

Milks Studied.—The milks examined were classified according to the number of bacteria contained, as follows: Class A, milks containing less than 10,000 bacteria per cubic centimeter (Samples 32, 35, 37, 23, 38, 25, and 24). Class B, milks with a count of between 10,000 and 100,000 bacteria per cubic centimeter (Samples 4, 3, 15, 29, 30, 6, 36, 10, 33, 31, 19, 39, and 12). Class C, milks with a count of between 100,000 and 1,000,000 bacteria per cubic centimeter (Samples 5, 11, 8, 28, 15, 27, 21, 26, 16, and 22). Class D, milks containing over 1,000,000 bacteria per cubic centimeter (Samples 1, 2, 18, 14, 20, and 17).

Bases of Comparison.—With both methods 3 counts were made. With the standard plates, these counts came from the different degrees of magnification used in counting the colonies. In comparing the two methods the count obtained by means of the highest magnification, when it was available in the standard plates, was compared with the count secured through the use of the low-power lens (16 mm.). These figures gave the most comparable results.

The milks belonging to Class A, with a bacterial count of from 0 to 10,000, gave the results recorded in Table 1.

Table 1 shows that counts obtained by the two methods parallel each other closely. The counts with both methods fell into the same class, and the order was the same in both cases, with the exception of Nos. 4, 5, and 6. The numbers obtained with the little plates were always larger, being on an average 60% higher. The ratio between the two counts varied from 1.10 to 2.86, as compared with 1. The percentage of variation between the results with the little plates of the same count was without exception less than the variation between the counts obtained with standard plates. This indicates that the results obtained by the rapid method are quite as reliable as those obtained by the standard-plate method, even tho somewhat higher.

² Am. Jour. Pub. Health, 1915, 5, p. 64.

TABLE 1
CLASS A, WITH COUNTS OF LESS THAN 10,000

	Serial Number	Standard Plates			Small Plates			Ratio Between Counts
		Number of Counts	Average Number of Bacteria per c.c.	Percentage of Variation	Number of Counts	Average Number of Bacteria per c.c.	Percentage of Variation	
1	34	4	675	25	4	945	14	1:1.25
2	32	6	940	48	6	1,060	20	1:1.14
3	35	6	2,625	60	6	2,925	8	1:1.11
4	37	5	2,900	64	6	5,165	16	1:1.78
5	23	2	3,000*	10	2	4,900	2	1:1.63
6	38	6	2,950	78	8	8,440	26	1:2.86
7	25	4	4,900*	19	4	9,500	5	1:1.94
8	24	2	8,300*	10	3	9,200	6	1:1.10
Average.....			3,286	39	...	5,267	12	1:1.60

* Plates counted with the naked eye only.

A comparative study of the milks with a bacterial count of from 10,000 to 100,000 on the standard plates gave the results shown in Table 2.

TABLE 2
CLASS B, WITH COUNTS BETWEEN 10,000 AND 100,000

	Serial Number	Standard Plates			Small Plates			Ratio Between Counts
		Number of Counts	Average Number of Bacteria per c.c.	Percentage of Variation	Number of Counts	Average Number of Bacteria per c.c.	Percentage of Variation	
1	4	2	15,500	3	2	8,200†	2	1:0.52
2	3	2	16,000	6	2	31,350	17	1:1.90
3	15	4	23,750	16	4	23,000	11	1:1.00
4	29	6	31,000*	35	2	99,000	5	1:3.12
5	30	4	34,000	36	2	75,000	0	1:2.20
6	6	3	34,000	50	2	20,000	0	1:0.58
7	36	2	40,000	25	3	13,400	11	1:0.32
8	10	4	41,000	23	4	22,000	12	1:0.53
9	33	4	42,500	30	4	42,400	11	1:1.00
10	31	6	46,000	25	4	50,000	12	1:1.08
11	19	4	51,000	35	3	14,800	17	1:0.27
12	39	6	75,000	37	3	72,630	7	1:0.96
13	12	3	96,700	73	4	17,000	21	1:0.17
Average.....			42,030	30	...	37,537	9.7	1:0.89

* Counted with naked eye only.

† Counted under the oil-immersion objective.

Table 2 records that the fluctuations were greater in this group than in the former. The ratio between the two counts varied from 0.17 to 3.12 as compared with 1. On the whole the number of bacteria found on the little plates was slightly less than the number found on the standard plates. The percentage of variation from the average count for the standard plates was higher than the percentage of variation

from the average for the little plates; that is, as 9.7 is to 30, or more than threefold.

The results for the milks the bacterial counts of which varied between 100,000 and 1,000,000 per cubic centimeter on standard plates are recorded in Table 3.

TABLE 3
CLASS C, WITH COUNTS BETWEEN 100,000 AND 1,000,000

	Serial Number	Standard Plates			Small Plates			Ratio Between Counts
		Number of Counts	Average Number of Bacteria per c.c.	Percentage of Variation	Number of Counts	Average Number of Bacteria per c.c.	Percentage of Variation	
1	5	4	110,000	45	1	28,300	0	1:0.25
2	11	4	129,500	65	4	35,500	45	1:0.27
3	8	4	148,000	18	3	206,000	9	1:1.32
4	28	5	166,000*	30	3	650,000†	17	1:3.31
5	13	3	171,000	23	2	225,000	12	1:1.31
6	27	5	293,000*	77	2	119,000	6	1:0.40
7	21	6	329,700*	18	4	610,000†	18	1:1.92
8	26	4	350,000*	27	2	103,000	3	1:0.29
9	16	4	474,000	10	4	134,000	2	1:0.29
10	22	3	903,000*	29	4	3,742,000†	16	1:4.14
Average.....			307,000	34	...	585,200	13	1:1.90

* Standard plates counted with naked eye only.

† Little plates counted with high dry power only.

In Table 3 it is seen that the comparative results were reasonably close. The ratio between the two counts varied from 0.25 (No. 1) to 4.14 (No. 10) as compared with 1. On the whole, the number of bacteria found by means of the little plates was higher than that found on the standard plates. The ratio was 1.9 for the little plates and 1 for the standard plates. The samples showing the greatest variation were those in which the data were incomplete; for example, Nos. 4, 7, 8, 9, and 10. It will be shown that the count on the standard plates is lower with the naked eye than with the lens, and that the count on the little plates is higher with a $\frac{1}{4}$ inch lens than with a $\frac{2}{3}$ inch lens, so that in these samples the data used tend to separate the samples, and may account for the high degree of variation. The percentage of variation from the average count was 34 in the case of the standard plates, and 13 for the little plates, indicating that the variation in the results from the different plates for each method is only one-third as great in the case of little plates as in the case of the standard plates.

The results obtained with milks varying in bacterial count from 1,000,000 to 10,000,000 per cubic centimeter (Class D) are given in Table 4.

TABLE 4
CLASS D, WITH COUNTS BETWEEN 1,000,000 AND 10,000,000

	Serial Number	Standard Plates			Small Plates			Ratio Between Counts
		Number of Counts	Average Number of Bacteria per c.c.	Percentage of Variation	Number of Counts	Average Number of Bacteria per c.c.	Percentage of Variation	
1	1	2	1,250,000*	12	2	1,160,000†	3	1:0.93
2	2	2	1,680,000	5	2	1,400,000	14	1:0.86
3	18	4	2,500,000	19	4	1,254,000‡	23	1:0.50
4	14	2	4,000,000	25	1	2,232,000	0	1:0.55
5	20	4	7,760,000	16	4	1,614,000†	9	1:0.20
6	17	4	20,750,000	37	2	15,990,000‡	2	1:0.72
Average.....			6,315,000	19	...	3,981,700	10	1:0.62

* Counted with naked eye only.

† Counted under oil-immersion objective.

‡ Counted under high dry power only.

The counts in this group demonstrate that the little plates give uniformly lower counts than the standard plates. The ratios between the two counts, No. 5 excepted, were very close, the average ratio being 1:0.62. The percentage of variation from the average was twice as great with the standard plates as with the little plates, that is, 19 to 10.

RESULTS AS A WHOLE

The results of the analyses recorded here are shown in the accompanying chart. The bacterial count, as determined by the standard-plate method is represented by the heavy continuous line. The maximal variations above and below the average are indicated by cross-hatching, accompanying the line, and extending up and to the right. The count of the same milks as determined by means of the little plates is shown by the broken line. The maximal variation from this average is represented by cross-hatching drawn down and to the right.

The scale at the left indicates the bacteria per cubic centimeter. Equal divisions on this scale represent an increase of tenfold at each horizontal line; i. e., in the lower tier the divisions equal 1,000, and in the upper tier 1,000,000. The numbers of the samples are indicated at the bottom of the chart.

On the whole, the correspondence seems reasonably close, and warrants the statement made in a previous communication, namely: "The results obtained indicate that the difference between the counts secured by the rapid method and the ordinary or standard method usually amounts to little more than the variation which occurs between duplicate plates, or between different dilutions in the same analysis by the ordinary plate method."³ Since only the comparative variations are

³ Frost: Science, 1915, 42, p. 255.

correctly visualized in the chart, the variations appear to be greater in Class A than in Class D, tho in reality they were smaller. The actual variations are recorded in the tables.

COUNTS AT DIFFERENT MAGNIFICATIONS

A. STANDARD PLATES

The number of bacteria obtained per cubic centimeter of milk varies with the magnification used in counting the colonies. This is in particular true of the little plates, tho it also applies to the standard plates.

Various counting devices are recommended by different workers. In Standard Methods for the Bacterial Examination of Milk⁴ it is recommended that "colonies too small to be seen with the naked eye or with slight magnification shall not be considered in the count." There are several counting apparatuses which use a lens of low power; e. g., a reading glass about 10 cm. in diameter. The committee on milk of the laboratory section of the American Public Health Association² proposes that the counting be done with a lens of $3\frac{1}{2}$ diameters magnification and recommends an engraver's lens (B. & L. 146).

Table 5 records the results which I have obtained using the different methods of counting.

TABLE 5
VARIATION DUE TO THE METHOD OF COUNTING STANDARD PLATES

Serial No.	Naked Eye	Reading Glass	$\times 3\frac{1}{2}$	Average Ratio Between Counts
Class A				
34	650	650	675	
32	625	880	940	
35	2,400	2,475	2,600	
37	2,500	2,800	2,900	
38	2,166	2,700	2,950	
25	4,800	4,800	4,900	
Average	2,190	2,384	2,494	1:1.09 : 1.14
Class B				
15	17,750	18,500	23,750	
30	16,250	18,750	34,000	
36	35,000	35,000	40,000	
33	37,500	41,500	42,500	
31	45,000	46,000	46,000	
19	42,000	43,000	51,000	
39	72,500	75,300	75,300	
12	58,000	91,700	96,700	
Average	40,500	46,220	51,156	1:1.14 : 1.26
Class C				
11	112,500	122,500	129,500	
13	142,000	161,000	170,000	
16	283,000	281,500	474,000	
Average	179,166	188,333	257,833	1:1.05 : 1.44
Class D				
18	1,870,000	2,125,000	2,500,000	
14	6,000,000	3,880,000	4,000,000	
20	6,740,000	7,150,000	7,760,000	
17	17,000,000	18,325,000	20,750,000	
Average	10,536,666	10,493,377	11,670,000	1:0.99 : 1.10

* Am. Jour. Pub. Hygiene, 1910, 6, p. 15.

In all cases save one (No. 14, Class D) the counts secured by the use of a lens magnifying $3\frac{1}{2}$ diameters were higher than those obtained with a lower magnification or with the naked eye. The variation is not great, however. If the average for naked-eye counts is taken as 1, the average for those made under the reading glass becomes 1.07 and for those made with the engraver's lens 1.23. The results obtained bear out the contention of the committee² on milk that if the true or maximal count is sought, counts made with a lens magnifying $3\frac{1}{2}$ diameters are more accurate than those made at lower magnifications. These higher counts have been selected, when possible, to compare with those obtained by the little plate method.

TABLE 6
COMPARISON OF COUNTS OBTAINED WITH THE RAPID METHOD AT VARIOUS MAGNIFICATIONS

Milk	Eyepiece 3 With			Average Ratio Between Counts
	Objective $\frac{3}{8}$ in. (82 \times)	Objective $\frac{1}{4}$ in. (200 \times)	Objective $\frac{1}{12}$ in. (840 \times)	
Class B	23,000	43,000	70,000	1 : 2.25:6.13
	99,000	200,000	290,000	
	75,000	54,500	104,000	
	20,000	112,000	200,000	
	22,000	44,000	125,000	
	42,400	128,500	745,000	
	50,000	96,000	125,000	
	14,800	58,700	87,000	
	72,630	188,000	793,000	
	17,000	56,000	132,500	
Average	43,580	98,070	267,150	
Class C	35,300	55,500	200,000	1 : 1.93:3.21
	206,000	260,000	430,000	
	225,000	234,000	480,000	
	119,000	490,000	600,000	
	103,000	400,000	600,000	
	134,000	152,000	335,000	
Average	137,050	265,250	440,833	
				1.00:2.09:4.67

B. LITTLE PLATES

The proper magnification in counting the little plates is a matter of importance because of the marked increase in count which followed the use of the higher powers, as demonstrated in Table 6. If the count obtained by the low-power 16-mm. lens be taken as 1, the 6-mm. lens ($\frac{1}{4}$ inch) gives a little over twice as many colonies, (2.09) and the oil immersion objective gives over $4\frac{1}{2}$ times as many (4.67). Two reasons for this disparity appear: The small colonies may be missed under the low power, and readily seen under the higher powers; and the high powers, especially the oil-immersion objective may reveal

groups of dead bacteria which cannot readily be distinguished from colonies. The low-power lens gives a count that, while too small, is the most comparable with that from the standard plates. The error due to the multiplication factor becomes smaller, offsetting the advantage of finer distinctions offered with the higher powers. A lens giving a magnification of 200 instead of 84 would seem the most desirable, and such a lens is now being tried out. The length of the incubation period of the colonies is important, because the older the colonies are, the larger they become and the less likelihood there is of confusing them with clumps of dead bacteria. Further work seems to show that when the period of incubation of the colonies in the little plates is from 6 to 8 hours, comparable counts are obtained with the different magnifications.

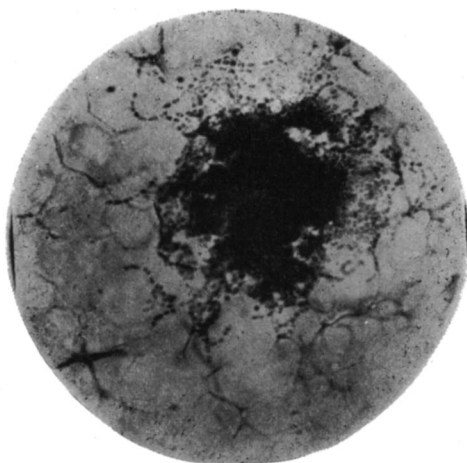


Fig. 3. A colony 5 hours old under the oil-immersion objective showing the individual cells and the arrangement during growth—probably *Bacillus acidilactici*. The reticulated structure of the medium is due to the fat globules in the milk which was used with the agar, which also accounts for the open places in the colony. $\times 750$.

PERIOD OF INCUBATION

The chief merit of, and the principal purpose in mind in developing this method of analysis was that it requires but a short period of incubation. Forty-eight hours are needed for the standard method. The period of incubation for the little plates may be reduced to 3 hours in some cases, 6 hours in most cases, and from 8 to 12 hours in all cases. Unless there is imperative need of reaching the results in less time, it is desirable to incubate not less than 6 hours in all cases. When the milk is fresh from the cow, or has just been pasteurized, it contains

very few bacteria, necessitating an incubation period of 12 hours, as the bacteria grow into visible colonies very slowly. With this limitation, the method can be used for the examination of certified and other milks with a low bacterial count. Market milks, or those having a high bacterial content, produce colonies in a remarkably short time, a 3-hour period of incubation being sufficient to obtain a large part of the colonies. A few extra hours for the incubation of the plates can be taken however even in such milks as these, indeed, they can also be left for 18 or more hours without any detriment except the formation of spreaders, which do not interfere with the use of the plates.

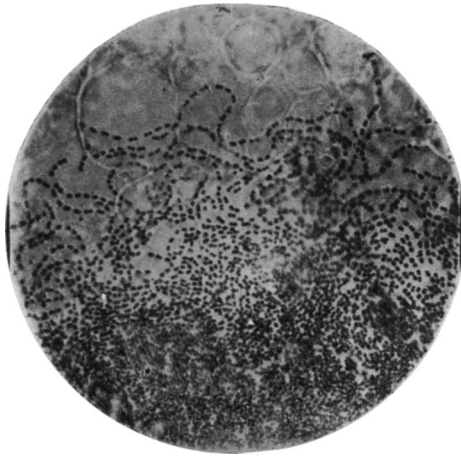


Fig. 4. The edge of a colony, showing individual bacteria and their arrangement in chains. This is probably a streptococcus colony. $\times 750$.

Most of the plates which were counted for the results recorded in this work were incubated for 5 hours or less, except those from the very good milk in Class A, which were incubated for 18 hours, altho 12 hours or less would have brought practically the same results.

ERRORS INHERENT IN THE METHODS OF ESTIMATING THE NUMBER OF BACTERIA IN MILK

In the standard method the chief sources of error lie in the dilution of the milk and the methods of counting. In the process of dilution many factors are variables,⁵ but even granting that all the factors in the process are constants, the high dilution to which milk must frequently be subjected, magnifies inherent difficulties in sampling, mea-

⁵ Frost: Tr. Wisconsin Acad. Sciences, 1914, 17, p. 1306.

suring, etc.; hence it is plain that bacterial counts of the same milk vary when the results are found by multiplying a small number of colonies actually counted by a high dilution factor. In addition the errors which must occur when a portion only of the colonies growing on a plate are counted, and counted under various degrees of magnification, further the divergencies in parallel determinations.

Some of these variable factors are avoided with the little plates. The dilution, for example, need not be over 1:200 as compared with the dilutions of 1:100,000, or 1,000,000 which are sometimes used. In the rapid method a new factor, already referred to as the microscope



Fig. 5. The edge of a colony found in milk showing a long square-end bacillus which grew very rapidly. $\times 750$.

factor, is present, and that is the quotient obtained by dividing the area of the little plates by the area of the microscopic field. For the microscope and magnification used ($\frac{2}{3}$ in. objective), this is 200; for the $\frac{1}{4}$ -inch objective 2,000, and for the oil immersion 20,000.

CULTURE MEDIUM

The amount of milk added to the agar, particularly in the lower dilutions, is large and changes the nature of the culture medium. This might serve to influence the bacteria which develop, and cause parallel counts obtained by different methods to diverge widely. However, there might be sufficient nourishment in the milk added to permit the substitution of a simple agar solution for the nutrient agar, thus simplifying the technic by obviating the making of culture media. In this

case, all dilutions of the milk to be analyzed would have to be made in sterile milk instead of water. As yet, no definite data on this point have been gathered except that results have not been such as to warrant a departure from the original technic of using nutrient agar.

DIFFERENTIATING BACTERIA BY MEANS OF THEIR MICROSCOPIC COLONIES

With the higher powers of the microscope the small colonies may readily be observed and the different micro-organisms recognized through their peculiarities, as shown by the photomicrographs. From a single preparation, then, the number of bacteria present can be determined, and individual micro-organisms recognized.

ADVANTAGES OF THE LITTLE-PLATE METHOD

The method is rapid, requiring only from one-twelfth to one-fourth of the time needed with the standard-plate method.

Its technic is simpler, requiring less glassware and culture medium.

It furnishes a means of keeping a permanent record since the slides can be filed.

By proper methods of staining, the individual bacteria or groups of the same that have not grown, can be seen and thus the total number of bacteria in the milk determined. This is not possible by the standard-plate method.

Then there is the advantage of being able to see whether or not the bacteria are alive, making it possible to study the germ content of pasteurized milk and to distinguish dead bacteria from the living, as cannot be done with the direct microscopic examination such as that recommended by Breed.

SUMMARY

Thirty-seven samples of milk varying in bacterial content from 675 to 20,000,000 bacteria per cubic centimeter were compared. Each sample was analyzed by the standard-plate and by the little-plate method, and a satisfactory correspondence between the results was found. With the exception of 2 samples, all were placed in the same classes by both methods. If the result obtained by the standard plates is taken as 1, the result, for the milks in Class A for the little plates, was 1.6; for Class B, 0.89; for Class C, 1.9; and for Class D, 0.62.

The results varied considerably with the different magnifications used in counting the colonies. Greater variations existed with the little

plates than with the standard plates, because under the higher powers groups of bacteria, dead or not yet grown into colonies, were counted.

The necessary period of incubation for the little plates varies from 3 to 12 hours.

A much larger amount of milk is used with the little plates than with the standard plates, hence the former method ought theoretically to be the more accurate of the two. The different counts on the same milk in the little plates always showed less variation among themselves than did those on the separate standard plates.

The rapid method permits the ready examination of young colonies of bacteria, and to this extent offers an important means of identifying particular bacteria.